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Glutaraldehyde treatment of allograft tissue decreases allosensitization after the Norwood procedure

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Objective: Cryopreserved allograft tissue used in the Norwood procedure for infants with hypoplastic left heart syndrome causes a marked immunologic sensitization that may complicate future heart transplantation. Treatment of the allograft tissue before implantation may prevent this sensitization. The purpose of this study was to assess the anti-human leukocyte antigen antibody response to glutaraldehyde-treated allograft tissue used in the repair of hypoplastic left heart syndrome.

Methods: Since June 2005, the University of Alberta has subjected allograft vascular tissue used in the Norwood procedure to glutaraldehyde treatment. An observational study was designed to assess whether glutaraldehyde treatment of the allograft tissue affected subsequent panel reactive antibody after patch implantation. Panel reactive antibodies for class I (human leukocyte antigen-A, B, C) and class II (human leukocyte antigen-DR, DQ) antibodies were measured 4 months postoperatively using flow cytometry.

Results: Fourteen patients underwent a Norwood procedure using glutaraldehyde-treated allograft tissue. Historical controls consisted of 12 patients who underwent a Norwood procedure using untreated allograft tissue. At 4 months, infants who had received glutaraldehyde-treated allograft tissue had lower class I panel reactive antibody (7.3% ± 17.4% [median, 0%] vs 61.9% [median, 73%] ± 39.9%; $P = .0005$) and class II panel reactive antibody (6.1% [median, 0%] ± 22.7% vs 49.3% [median, 63%] ± 41.9%, $P = .001$) compared with the historical controls.

Conclusion: Intraoperative glutaraldehyde treatment of allograft tissue used in hypoplastic left heart syndrome repair prevents the profound immunologic sensitization that occurs in the majority of infants undergoing surgical palliation. In patients requiring subsequent heart transplantation, this decreases the risk of antibody-mediated rejection and increases the likelihood of finding a suitable donor, thus improving access to transplantation. (J Thorac Cardiovasc Surg 2010;139:1402-8)

Although the results of the Norwood operation for hypoplastic left heart syndrome (HLHS) have improved over the past 2 decades, long-term outcomes are still unknown and a proportion of these children will eventually require cardiac transplantation.[1] Human allograft, or ''homograft,'' tissue is routinely used for aortic reconstruction during the Norwood procedure. Allosensitization has been identified as a consequence of allograft tissue use in cardiac surgery.[2,3] This sensitization can be measured as panel-reactive antibody (PRA), which describes the proportion of potential donors in a community against whom the patient has preformed antibodies. Outcomes after transplantation in sensitized patients are inferior to unsensitized patients.[4] Patients receiving a ''rescue'' cardiac transplant after palliative HLHS repair have a survival disadvantage compared with patients undergoing primary cardiac transplantation for HLHS. This survival disadvantage is due to allosensitization.[5] In addition to inferior outcomes, sensitized patients are subject to longer wait times and higher mortality while waiting for transplantation. Thus, a method to decrease the sensitization that results from allograft tissue use is crucial.

Glutaraldehyde is an aldehyde fixative often used in tissue preservation. Porcine and bovine tissue can be treated with glutaraldehyde to allow xenotransplantation in the form of valved conduits. The effective decellularization and tissue-strengthening properties of glutaraldehyde, as well as its ability to cross-link antigens, make xenograft tissue resistant to both immune and nonimmune-mediated destruction.[6] We hypothesized that glutaraldehyde treatment of allograft tissue would have a similar effect. Thus, the purpose of this current study was to determine whether glutaraldehyde could prevent the sensitization response induced by exposure to allograft tissue patches used in the repair of HLHS.

MATERIALS AND METHODS

Study Design

An observational cohort study was conducted to compare the effect of glutaraldehyde treatment of cryopreserved allograft tissue on PRA after

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Abbreviations and Acronyms
cPRA = calculated panel reactive antibody
HLA = human leukocyte antigen
HLHS = hypoplastic left heart syndrome
PRA = panel reactive antibody

the Norwood procedure for HLHS. PRA was assessed at 4 months postoperatively. The study was approved by the Human Research and Ethics Board at the University of Alberta. Cryopreserved aortic homografts were treated separately with glutaraldehyde and stained for class I and II human leukocyte antigen (HLA) to detect differences in antigen expression before and after treatment.

Study Cohort

Fourteen infants born with HLHS underwent first-stage palliation (Norwood procedure) involving aortic arch reconstruction using glutaraldehyde-treated cryopreserved pulmonary artery allograft tissue between June 2005 and June 2007. Before implantation, the valved allografts were treated with a glutaraldehyde solution and subsequently rinsed in normal saline and fashioned in the shape needed to reconstruct the neo-aorta. Early in the series, a 2.6% glutaraldehyde solution was used, but this was modified to a 1% glutaraldehyde solution in 0.9% normal saline. In short, the valved pulmonary artery homograft was thawed and submersed in a 1% glutaraldehyde solution for 10 minutes in the operating room under sterile technique. A sponge was often placed inside the lumen of the homograft to help maintain its shape during fixation. The allografts were then rinsed in 0.9% normal saline for 10 minutes. The nonvalved portion of the allograft tissue was then shaped accordingly and implanted into recipients. Thirteen allografts were provided by the Comprehensive Tissue Center at University of Alberta (Edmonton, Alberta, Canada), and 1 allograft was provided by Cryolife Inc (Kennesaw, Ga).[7] Peripheral blood samples were collected 4 months after implantation when the patients returned for second-stage surgery.

Control Cohort

Twelve infants who underwent aortic arch reconstruction (Norwood procedure) for HLHS at the University of Alberta between February 2003 and September 2004 (before the use of glutaraldehyde-treated of allograft tissue) acted as a historical control cohort. These patients were involved in a prior study at the University of Alberta, and no new blood samples were drawn.[2] Historical data from this cohort were used with permission of the authors.

Variables Studied

Preoperative variables that ensured similarity between the 2 groups included age, gender, preoperative length of hospitalization, and blood product exposure. Perioperative factors included duration of aorta crossclamping and cardiopulmonary bypass, use of hypothermic circulatory arrest, and blood product exposure. Postoperative variables included length of stay in intensive care unit, length of stay in hospital, and blood product exposure. All infants received cytomegalovirus-negative, leukocyte-depleted blood.

Donor and Recipient Human Leukocyte Antigen Typing

Donor and recipient class I and II HLA typing was performed by molecular methodology. Recipient DNA was purified from whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, Calif). Donor DNA was purified from blood, and if no blood was available, bone marrow tissue was collected by the Comprehensive Tissue Center. HLA-A, B, and DR antigen typing was performed using the low-resolution Micro SSP DNA typing kit (One Lambda, Inc, Canoga Park, Calif). DNA fragments were separated by agarose gel electrophoresis. HLA antigens were determined through a combination of One Lambda DNA/LMT software analysis and manual interpretation of the electrophoresis results.

Human Leukocyte Antigen Antibody Analysis

Screening for anti-HLA antibodies and antibody specificity for class I and II HLA was performed as previously described.[2] In short, the Flow PRA Screening Test (One Lambda) was used, which involved exposing patient sera to a mixture of beads coated in class I and II antigens. Bound antibody was detected with fluorescein-isothiocyanate conjugated anti-human immunoglobulin-G $F[ab']_2$ and measured on a FACSCalibur flow cytometer (BD Biosciences, San Jose, Calif). Samples that were positive (PRA > 5%) were tested for antibody specificity using single-antigen beads (Catalog FL2HD and FL1HD; One Lambda). Calculated PRA (cPRA) values were calculated using the United Network for Organ Sharing cPRA calculator.[8,9]

Immunohistochemistry of Cryopreserved Allografts

Cryopreserved aortic allografts were separately collected from the Comprehensive Tissue Center (Edmonton, AB, Canada). These allografts consisted of stock that was discarded because of expiration or defects preventing their use clinically. These allografts were procured and stored identically to clinically used allografts. The vessel wall of the allograft was cut into 1-cm sections and exposed to glutaraldehyde solutions and stored in optimal cutting temperature compound at –80°C. After cryosectioning, residual aldehydes were quenched with 0.1 mol/L glycine. Samples were then stained using standard immunohistochemistry techniques with biotinylated secondary antibodies, a peroxidase avidin/biotin complex, and 3,3'-diamniobenzidene as the chromogen. Samples were counterstained with hematoxylin. Class I HLA was detected with anti-HLA ABC (Catalog 311402; BioLegend, San Diego, Calif), and class II HLA was detected with anti-HLA DR (Catalog 307602; BioLegend).

Statistical Analysis

Statistical analysis was performed using STATA 9.1 (StataCorp, College Station, Tex). Nonparametric tests were used including Wilcoxon rank-sum test for continuous data and Fisher's exact test for binomial data. Data are expressed in mean and standard deviation for parametric data and median and standard deviation for nonparametric data.

RESULTS

Patient demographics and operative information are summarized in Table 1. With the exception of age at surgery and interval between surgery and PRA measurement, the groups are well matched. The more recent cohort was slightly older at the time of the surgery (8.3 ± 4.8 days vs 14.7 ± 9.7 days; $P = .05$) with a slightly longer time period between surgery and PRA measurement (124.7 ± 16.5 days vs 152.4 ± 40.1 days; $P = .04$). This most likely reflects a change in practice as we have become more comfortable extending the operative window from the first week of life.

There was a significant decrease in the class I PRA response resulting from exposure to the allograft material in the glutaraldehyde-treated tissue group compared with the control group (7.3% [median, 0%] ± 17.4% vs 61.9 [median, 73%] ± 39.9%; $P = .0005$) (Figure 1). There were only 2 patients (14.2%) who had a class I PRA greater than 10% compared with 11 patients (83%) in the historical

TABLE 1. Patient characteristics

Variable	Control	Glutaraldehyde group	*P* value
N	12	14	
Gender (m)	75%	50%	.24
Age at surgery (d)	8.3	14.7	.05
Bypass time (min)	127	111	.45
Aortic crossclamp time (min)	42.6	46.8	.64
Circulatory arrest time (min)	28	23.5	.27
Height (cm)	50.5	51.5	.37
Weight (kg)	3.4	3.3	.81
Blood (units)	7.4	6	.60
Platelet (units)	4.8	2.6	.35
Fresh-frozen plasma (units)	1.3	0.4	.11
Interval between surgery and PRA (d)	124.7	152.4	.03

PRA, Panel reactive antibody.

cohort. The glutaraldehyde-treated patch group also had significantly decreased class II PRA compared with the historical cohort (6.1% [median, 0%] ± 22.7% vs 49% [median, 63%] ± 41.9%; $P = .001$). There was only 1 patient (7%) in the glutaraldehyde-treated patch group who had a class II PRA more than 10%, whereas there were 9 patients (75%) in the control group (Figure 1). There was 1 patient who was positive for both class I and II in the glutaraldehyde-treated patch group; in contrast, there was only 1 patient in the control cohort who was negative for both class I and II PRA. A PRA of more than 10% in either class I or II is considered clinically significant sensitization.[10] By reorganizing the data into clinically significant and clinically insignificant PRA based on this value, the effect of glutaraldehyde-treatment persists ($P < .001$) (Table 2). cPRA describes the percentage of donors who would have 1 or more unacceptable antigens for a sensitized recipient. This calculation is based on previous donor typings and listing potential

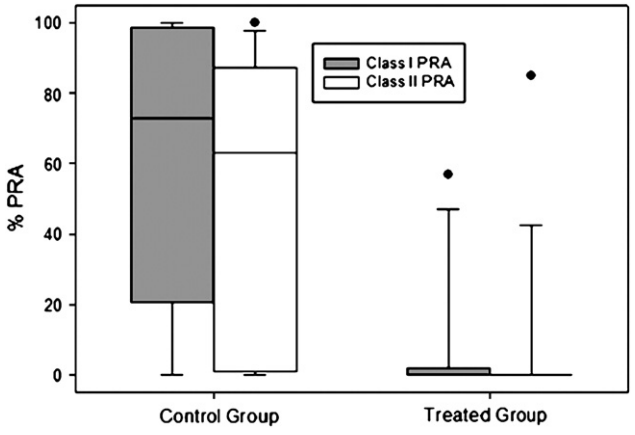

FIGURE 1. Box-plot of PRA by group. The median, 25th, and 75th percentiles are shown. Outliers are expressed as separate *dots* outside the upper fence. *PRA,* Panel reactive antibody.

TABLE 2. Class I or II panel reactive antibody less than or more than 10%

PRA	Control	Glutaraldehyde group	Total
<10%	1	12	13
>10%	11	2	13
Total	12	14	26

PRA, Panel reactive antibody.

donor antigens as unacceptable (Table 3). Using cPRA confirms the prevention of a significant sensitization response in the glutaraldehyde treatment group (65% ± 37.3% in historical cohort vs 8.9% ± 26.0% in glutaraldehyde-treated group; $P < .0001$). No significant differences in PRA were detectable between patients who had received grafts treated with 1% or 2.6% glutaraldehyde solutions.

In an attempt to identify the source of sensitization, antibody specificity was analyzed. This confirmed that many of the antibodies generated were specific for the HLA type of the donor allograft (Table 3). Equally important was the failure to generate anti-HLA antibodies in the absence of HLA mismatch in individuals who were coincidentally HLA-matched with their donor allograft. Patient 7 had no mismatched class I HLA antigens on the untreated allograft they received and failed to generate any class I antibodies.

Because blood transfusions can contribute to sensitization, linear regression analysis was performed to detect a possible relationship between number of blood unit exposures and class I and II PRA. There was no identifiable relationship between blood transfusion and PRA (class I PRA: $\beta = -1.29$, $R^2 = 0.04$, $P = .30$; class II PRA: $\beta = -0.60$, $R^2 = 0.01$, $P = .62$).

There were no adverse outcomes associated with the treatment of allograft patches with glutaraldehyde before implantation. Incidentally, there was note of subtle patch calcification at the time of the second-stage surgery at 4 months. Specifically, there was no recurrence of aortic coarctation in the glutaraldehyde-treated group.

Immunohistochemistry analysis revealed less detectable HLA on the allograft tissue after glutaraldehyde treatment (Figure 2). Furthermore, with increased exposure time or glutaraldehyde concentration, a dose-response relationship emerged. This is qualitatively expressed in Table 4.

DISCUSSION

Implantation of cryopreserved allograft tissue has been shown to trigger an immune response, despite the previous assumption that it was immunologically privileged.[3] The immune response elicited could shorten the life of valved allografts used for valvular replacement.[11] Exposure to single-donor HLA results in a much broader sensitization than expected because of the high level of cross-reactivity between these antigens. Such cross-reactive epitope groups explain most of the non–donor-directed antibodies found.

TABLE 3. Donor-recipient human leukocyte antigen mismatch and antibody specificities at 4 months

Patient	Antibody specificities	Donor-specific antibodies	PRA 4 mo		cPRA%
			Class I (%)	Class II (%)	
1	None	None	0	0	0
2	A2 A11 A23 A24 A25 A29 A30 A31 A32 A33 A34 A68 B7 B13 B27 B44 B45 B49 B55 B57 B60 DR7 DR9 DR11 DR12 DR13	No donor typing	100	59	98
3	N/A	N/A	92	0	N/A
4	A1 A11 A29 B8 B27 B44 B45 DR7 DR9 DR17	A1 A29 B8 B44 DR7 DR17	99	82	72
5	None confirmed	None	17	0	N/A
6	A23 A24 B21 (B49 B50) DR13 DR17 DR53 DR103	A24 B50 DR13	60	93	83
7	DR1 DR15	DR1 DR15	0	67	39
8	A2 A11 A23 A24 A25 A26 A30 A68 B57 B65 DR13 DR14 DR17	A2 B14 (65) DR13	65	85	92
9	A3 A11 A30 A31 B13 B35	A3 B35	31	3	53
10	A3 A24 A25 A29 A32 A33 B8 B13 B18 B27 B35 B40 B45 B49 B55 B57 B62 B65 DR1 DR9 DR11 DR13 DR15 DR103	A24 B8 B40 DR1 DR13	98	99	97
11	A1 A3 A11 A23 A24 A25 A31 A32 B27 B49 B52 B57 DR1	A11 B27 DR1	81	15	82
12	A3 A23 A24 A25 A29 A30 A31 A32 A33 A34 A68 B8 B13 B18 B27 B38 B44 B45 B49 B51 B52 B57 B62 B65 DR7 DR9 DR11 DR12 DR13	No donor typing	100	100	99
13*	B7 B27 B45	B27	37	0	30
14*	A2 A24 B7 B27 DR1 DR 10 DR 11 DR15 DR103 DR51 DR53	A2 B7 DR1 DR15 DR51	57	85	95

PRA, Panel reactive antibody; *cPRA,* calculated panel reactive antibody. *Glutaraldehyde-treated group. Patients 1 to 12 consist of the historical control cohort and patients 13 and 14 represent the sensitized patients in the glutaraldehyde-treated group.

For example, in patient 2 (Table 2) only the antibodies to HLA-A1, A29, and B27 are donor related. However, HLA-A11 and HLA-A1 share a public epitope. Similar cross-reactivity exists among HLA-B27, B49, B52, and B57. Conversely, in the patient matched for class I antigens, there was no class I PRA response. Blood transfusions are known to be potentially sensitizing, but we observed no relationship between blood transfusions and PRA in this study. This is consistent with previous work at the University of Alberta involving a group of infant patients not

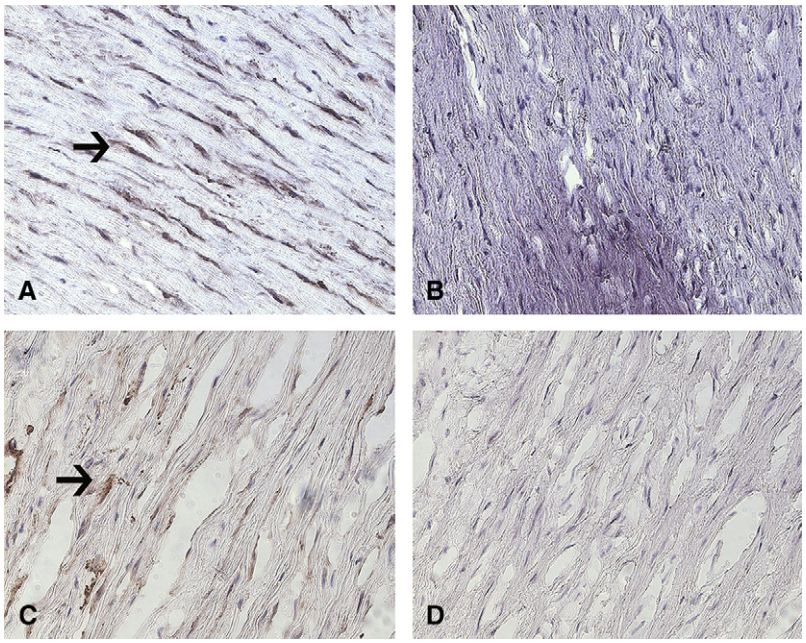

FIGURE 2. Immunohistochemistry of aortic allograft. A, Untreated aortic allograft stained for HLA-ABC. B, Glutaraldehyde-treated allograft stained for HLA-ABC. C, Untreated aortic allograft stained for HLA-DR. D, Glutaraldehyde-treated allograft stained for HLA-DR. Positive staining (*arrows*).

TABLE 4. Effect of different concentrations and exposure times of glutaraldehyde on human leukocyte antigen expression

HLA-ABC					HLA-DR				
	Exposure time					Exposure time			
Concentration	5 min	10 min	15 min	20 min	Concentration	5 min	10 min	15 min	20 min
0.25%	+	+	+	+	0.25%	+	+	+	+
0.5%	+	+	-	-	0.5%	+	+	-	-
1.0%	+	-	-	-	1.0%	+	-	-	-
2.0%	-	-	-	-	2.0%	-	-	-	-

HLA, Human leukocyte antigen. ''+'' indicates presence of staining and ''-'' indicates absence of staining.

exposed to allograft tissue but who received blood transfusions.[2] Whether this is due to the age of the recipients, the transient nature of the blood transfusion, or the possible immunomodulating effects of the blood transfusion itself is unknown.

Allosensitization that occurs in the neonatal period from allograft tissue persists for at least 8 years, if not indefinitely.[12] Transplantation can be used a ''rescue'' should it be required during the palliative repair steps of HLHS. Successful palliation includes a lifelong risk of Fontan failure.[5] In the setting of organ transplantation, a sensitized patient faces certain disadvantages. The presence of preformed anti-HLA antibodies is associated with increased rates of early high-grade rejection and graft vasculopathy.[13] As a result, survival after transplantation is clearly diminished.[4] Thus, sensitized patients may be excluded from transplantation or limited to rarely available HLA-matched local donors. This is true across different types of organ transplantation. Enormous effort is currently being directed toward the identification and management of these sensitized patients.[14] PRA values depend on the assay and the HLA panel used, and therefore variability can be found between centers.[15] The detection of HLA-specific antibodies allows identification of unacceptable antigens, and a more clinically relevant ''PRA value'' can be calculated. The cPRA reflects the potential availability of an organ, in particular the donor pool for a given sensitized patient. This calculator is based on the kidney donor population, but the heart donor pool may be considerably smaller. cPRA correlates well with positive crossmatch outcomes.[8]

Attempts to prevent immune sensitization have been largely unsuccessful. Intravenous immunoglobulin has been used with variable success in ''desensitizing'' protocols to decrease antibody levels to allow renal transplantation.[16] However, when used in the setting of a Norwood surgery, intravenous immunoglobulin was ineffective at preventing sensitization.[17] Although mycophenolic mofetil can reduce the HLA antibody response after allograft tissue use, lifelong immunosuppression is undesirable, especially in the pediatric population.[18] Decellularization has been used repeatedly in animal studies to prevent generation of donor-specific antibodies.[19] Efforts are being made to repopulate such decellularized tissue with host cells, but such techniques await advances in stem cell research. HLA matching of donor and recipient would theoretically prevent sensitization as well. Only recently has our local comprehensive tissue center begun HLA typing of tissue donors. Although possible, lack of sufficient allograft donor tissue would make it difficult to match all patients.

Glutaraldehyde has been used in cardiac surgery for decades. Xenograft valves are routinely treated with glutaraldehyde because it is thought to prevent xenograft rejection.[20] Glutaraldehyde is also used to strengthen autologous pericardium when it is used for patching cardiac defects. Cryopreservation alone removes many of the endothelial cells.[21] When implanted valved allografts are removed surgically during subsequent cardiac surgical procedures, no donor cellular elements remain.[22] As shown by Baskett and colleagues,[11] allograft cellular viability may promote early graft failure. Thus, removal of cellular elements from these tissue grafts may be desirable. As an aldehyde fixative, glutaraldehyde has the potential to decrease the cellular viability of allograft tissue.

Glutaraldehyde-treated tissue tends to calcify when placed in vivo. Xenografts undergo anti-calcification treatments such as with α-amino-oleic acid to help minimize this in the setting of biological valve replacement, yet they still eventually fail from calcific degeneration.[23] Many, if not most, allograft tissue transplants calcify in the absence of glutaraldehyde treatment, and this calcification is more pronounced in young children.[24] The enrolled patients who have returned for the second-stage operative palliation for HLHS at the University of Alberta have had noticeably more calcification of the neoaortic patch if it had been treated intraoperatively with glutaraldehyde. Because the patch extends to the site of aortic coarctation repair, there is a theoretic risk of increased coarctation recurrence after use of a glutaraldehyde-treated patch. Recurrence of aortic coarctation does occur after the Norwood procedure regardless of patch material used; however, we have observed no cases of re-coarctation in the patients enrolled. It is also possible to speculate that treatment with glutaraldehyde might reduce the incidence of coarctation by reducing the recipient immune response to the patch. Aortic calcification can complicate reoperative surgery. To date, there has been no difficulty during cannulation of these aortas at the 4-month

bidirectional cavopulmonary shunt creation or during subsequent Fontan creation.

The immunohistochemistry results suggest that the mechanism of action of glutaraldehyde is disruption of the HLA molecules on the allograft tissue itself. Whether this is from accelerated cell death, decreased HLA production in remaining viable cells, or alteration of the HLA complex itself is unclear. Aldehyde fixatives cross-link proteins and frequently mask antigens when used for tissue fixation for histologic assessment. This antigen ''masking'' could be the very mechanism preventing immune sensitization. Antigen retrieval steps during immunohistochemical staining can be used to reverse this masking. In our immunohistochemistry protocol, fresh-frozen tissue and cryosections were used after glutaraldehyde treatment to avoid the need for antigen retrieval. By immunohistochemistry, expression of both class I and II HLA were absent at a concentration of 1% glutaraldehyde with exposure times of 10 minutes. This correlates with the observation that there was no difference in the PRA response between patients with allograft tissue treated with 1% versus 2.6% glutaraldehyde in our protocol. However, the patches treated with 2.6% glutaraldehyde may be subject to more calcification. Although staining with a monoclonal antibody cannot replicate the sensitivity of in vivo anti-HLA antibodies produced in a sensitized recipient, it does suggest a dose-dependent decrease in the availability of HLA with glutaraldehyde treatment. Of note, xenografts are typically treated for many days with glutaraldehyde, in contrast with the 10-minute treatment used in this study. There was 1 patient in the treatment group who was a statistical outlier with a very high class I and II PRA. Because glutaraldehyde is colorless and odorless, it is possible that this patient's allograft was mistakenly not treated with glutaraldehyde.

Limitations

Our study has several limitations. Cohort studies can be subject to bias, and it was deemed impractical to do a randomized clinical trial in this setting. HLHS is an uncommon lesion even at large referral centers. The allografts used in this study all consisted of non-valved allograft wall only; therefore, these results should not be extrapolated to valved allograft conduits in which valve function needs to be considered. The effect of tissue treatment was not universal or absolute in all subjects, suggesting there are other factors specific to both the recipient and the donor tissue that may prove to be important. Because of the lack of available donor lymphocytes, we were unable to assess the cell-mediated immune response and only comment on humoral immune factors involved. The increased calcification noted in the glutaraldehyde-treated allograft tissue group was not fully anticipated and as a result not measured objectively. The calcification of this tissue may continue past the 4-month follow-up period.

CONCLUSIONS

Treatment of allograft tissue with glutaraldehyde can prevent the sensitization associated with cryopreserved allograft use after neoaortic reconstruction in HLHS surgery. Glutaraldehyde treatment is easy to perform in the operating room with few potential disadvantages. This simple technique has dramatic potential to improve outcomes for these individuals who require subsequent heart transplantation.

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